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Isolation and Characterization of Monoclonal Antibodies Specific for Protein Conformational Epitopes Present in Prostate-Specific Membrane Antigen (PSMA)

WILLIAM T. TINO,[1] MONICA J. HUBER,[1] THOMAS P. LAKE,[1] THOMAS G. GREENE,[1] GERALD P. MURPHY,[2] and ERIC H. HOLMES[1]

ABSTRACT

Prostate-specific membrane antigen (PSMA) is a 750–amino acid glycoprotein highly expressed in malignant prostate tissues. PSMA reacts with the murine monoclonal antibody 7E11.C5, whose binding epitope has been mapped to the *N*-terminal of the protein distributed on the cytoplasmic side of the plasma membrane. We have developed murine monoclonal antibodies specific for extracellular epitopes of PSMA. Three of these antibodies—1G9, 3C6, and 4D4—display distinct binding properties consistent with their recognition of conformational epitopes within native PSMA. Results indicate this panel of antibodies binds to native full-length PSMA, but not to fusion proteins containing portions of the linear sequence of the protein. Antibody binding is greatly reduced upon heat denaturation of native PSMA, and these antibodies do not detect PSMA by Western blot. Immunoprecipitation experiments demonstrate the ability of each to bind to full-length PSMA as well as PSM', a form of the protein missing the first 57 amino acids. These results indicate each antibody is specific for an epitope within the extracellular domain, a region spanning residues 44–750. Flow cytometric experiments indicate strong specific binding to live LNCaP cells. Antibody inhibition studies demonstrate that these antibodies recognize at least two distinct epitopes. Taken together, the results demonstrate that these antibodies are specific for native protein conformational epitopes within the extracellular domain. Their properties, in particular strong binding to live cancer cells, make them ideal candidates that are clearly superior to linear sequence epitope specific antibodies for *in vivo* applications.

INTRODUCTION

PROSTATE-SPECIFIC MEMBRANE ANTIGEN (PSMA) is a 750–amino acid type II transmembrane glycoprotein of approximately 110 kDa.[1,2] Its expression has been extensively studied by several investigators.[3–13] These results indicate that, at the protein level, PSMA is highly restricted to prostatic epithelial cells. Expression of PSMA is upregulated in prostatic cancer, particularly in hormone refractory disease.[14–16] Recent immunohistochemical studies[4–3] confirm the increased PSMA expression in prostatic tumors.

Extraprostatic expression of low levels of PSMA protein has been found in tissues such as brain, salivary gland, and duodenum.[7–10] Interestingly, PSMA has also been detected in endothelial cells of blood vessels present in a variety of cancerous tumors, but not in blood vessels from normal tissues.[11,12] This expression pattern suggests that PSMA is a useful marker for prostatic cancer and perhaps has application in other cancers.

PSMA has three structural domains, including a 19–amino acid intracellular domain, a 24–amino acid transmembrane domain, and a 707–amino acid extracellular domain. PSMA was originally defined by the monoclonal antibody 7E11.C5.[3] Subsequent studies indicated that this antibody targets an intracellular epitope composed of the first six amino acids from the *N*-terminus of the protein.[17] Additional monoclonal antibodies specific for extracellular portions of the PSMA protein have been described.[11,12,18–20] In general, these antibodies appear to be specific for linear sequence epitopes distributed throughout the extracellular domain, and have been utilized in studies of tissue localization of PSMA and as a possible therapeutic reagent.[21] As a part of our PSMA antibody development pro-

[1]Northwest Biotherapeutics, Inc., Seattle, Washington.
[2]Pacific Northwest Cancer Foundation, Seattle, Washington.

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gram, we have obtained a small number of hybridomas that secret antibodies with distinct antigen binding properties. Results presented in this paper indicate that these antibodies are most probably specific for conformational protein epitopes present in the extracellular domain, and not epitopes defined by a linear amino acid sequence.

The high prostate tissue specificity of PSMA and the effectiveness of these antibodies in binding to live PSMA expressing cells suggests that they may have considerable utility in clinical diagnostic and therapeutic applications.

MATERIALS AND METHODS

Materials

LNCaP and PC-3 cells were obtained from the American Type Culture Collection (Rockville, MD). The monoclonal antibody 7E11.C5 was obtained from Cytogen, Inc. (Princeton, NJ). Freund's adjuvant was obtained from Gibco-BRL (Rockville, MD). NP-40 was obtained from Sigma (St. Louis, MO). Triton X-100R was obtained from Aldrich (Milwaukee, WI). Extractigel-D and Immunobeads were obtained from Pierce (Rockford, IL). Bacterially expressed T7 fusion proteins containing segments of PSMA were provided by Dr. Mo Saedi, Hybritech/Beckman (San Diego, CA). All other reagents were of the highest purity commercially available.

Methods

Immunization and fusion of mice. Adult female Balb/c and A/J mice were initially immunized intraperitoneally with LNCaP cell membranes in Freund's complete adjuvant. Subsequent immunizations at 2- to 3-week intervals utilized Freund's incomplete adjuvant and LNCaP cell membranes (one boost) and three boosts with 50 μg each of purified LNCaP cell-derived PSMA. PSMA was purified from PBS containing 1% NP-40 lysates of LNCaP cells by immunoaffinity chromatography on a 7E11.C5-Immunobeads column with elution by 100 mM glycine buffer, pH 2.5, containing 1% Triton X-100R. Detergent was removed by passage of the eluted fractions over an Extractigel-D column, and the protein was lyophilized and dialyzed extensively with PBS prior to use in immunizations or hybridoma screening. Spleen cells were fused with P3X63Ag8U.1 mouse myeloma cells at a ratio of 1:1 according to established procedures.[22]

Solid phase ELISA. A solid-phase enzyme-linked immunoadsorbent assay (ELISA) was employed for the detection of PSMA-specific antibodies. Immunoaffinity purified PSMA from LNCaP cells, or bacterially expressed fusion proteins containing PSMA derived fragments were coated onto Maxi-Sorp (Nunc, Rochester, NY) 96-well plates with an overnight incubation at 4°C. The plates were washed with phosphate-buffered saline (PBS: 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4)/0.2% Tween-20 and blocked with 5% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Fifty microliters of supernatant from the hybridoma cultures was added to the PSMA-coated wells, and the plates were incubated for 2 h at room temperature. The plates were washed as above and 50 μL of 1:600 diluted rabbit-anti-mouse IgG and rabbit-anti-mouse IgM (ICN, Costa Mesa, CA) was added to each well. Following a 1-h incubation at room temperature, the plates were washed as above and 50 μl of a 1:400 dilution of HRP-conjugated Protein-A (Sigma) was added to each well. Following a 1-h incubation at room temperature, the plates were washed as above and 100 μL of ABTS (150 mg 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid in 500 mL of 0.1 M citric acid, pH 4.35]/H_2O_2 (10 μL 30% H_2O_2 per 10 ml of ABTS solution) chromagen/substrate solution was added to each well. After a 5-min incubation, the reaction was stopped with the addition of 100 μL of stop solution (SDS/dimethylformamide) and the absorbance at 405 nm was read in a microplate reader. The hybridoma cells producing supernatants with high A^{405} values were cloned by limiting dilution and subjected to additional analysis.

For solid-phase capture of PSMA, this assay was modified as follows. Fifty microliters of 0.1 M $NaHCO_3$, pH 8.2 binding buffer containing 40 μg/mL of 4E10-1.14 anti-PSMA IgM antibody purified from ascites fluid was added to wells of a Maxi-Sorp plate and allowed to adhere overnight at 4°C. The plates were washed and blocked as described previously. Fifty microliters of serially diluted immunoaffinity-purified PSMA was added to the wells, and the plates incubated for 2 h at room temperature. Following extensive washing, 50 μL of undiluted tissue culture supernatant of anti-PSMA IgG antibodies was added to the wells and the plates were incubated for 90 min at room temperature. After washing the plates as above, the plates were incubated for 1 h at room temperature with 1:2,000 diluted HRP-conjugated rabbit-anti-mouse IgG (Fc fragment specific; ICN). The amount of anti-PSMA IgG antibody bound to the plates was then determined as described previously.

Isolation of antibody protein

Monoclonal antibodies were isolated from a Cellmax bioreactor (Cellco, Laguna Hills, CA) using HyQ-CCM1 medium containing 1–5% Fetalclone (Hyclone, Logan, UT). Monoclonal antibodies were purified by chromatography on a Protein G–Agarose column according to manufacturer's specifications (KPL, Gaithersburg, MD).

Preparation of LNCaP cell membranes

LNCaP cells were scraped from plastic dishes, washed extensively in PBS, resuspended in 10 volumes of deionized water, and homogenized by three strokes with a Dounce homogenizer. The membrane fraction was isolated by centrifugation at 15,000 × *g* for 45 min and the pellet resuspended in PBS. Protein concentration of the membrane pellet was determined using the Pierce BCA kit.

Heat denaturation experiments

An aliquot of immunoaffinity purified PSMA from LNCaP cells (40 μg/mL in PBS) was heat denatured by boiling for 10 min and cooled on ice. This, and an identical aliquot which was not heat denatured, were diluted 1:4 in PBS and 50 μL was added to wells of a 96-well Maxi-Sorp plate (Nunc) and coated overnight at 4°C. Alternatively, an aliquot from an LNCaP cell membrane preparation (80 μg/mL in PBS) was heat denatured

and cooled. This, and an identical aliquot without heat denaturation, was diluted 1:4 in PBS and 50 μL was added to a 96-well plate. Membranes were dried onto the plate by placing the plate on a 37°C warming plate overnight. After coating, all plates were blocked with 5% BSA in PBS for 1 h, washed in PBS, and subjected to a standard sandwich ELISA using the indicated primary antibodies as described above.

Western blot analysis

Western blot analysis was performed following sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of PSMA containing fractions and transfer to PVDF membranes. The blots were blocked overnight in TBS containing 5% nonfat milk and incubated with purified antibody present at a concentration of 5 μg/mL in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 1 h. The blots were washed five times with TBS containing 0.5% Tween-20 (TBS-T) and incubated with 1:5,000 HRP-conjugated goat-anti-mouse IgG for 1 h. After five washes with TBS-T, the blots were developed using the LumiGLO chemiluminescent substrate kit (KPL, Gaithersburg, MD) and visualized by exposing x-ray film.

Immunoprecipitation studies

A detergent lysate of LNCaP cells was prepared by adding PBS containing 1% NP-40 to LNCaP cells, incubating for 1 h, and centrifugation to remove particulate material. The lysate was precleared by adding 150 μg irrelevant human IgG_1 per mL of lysate, incubating for 1 h at room temperature, followed by the addition of 150 μL of packed Protein G-Sepharose beads per ml of lysate. The supernatant fraction was used after centrifugation to remove the beads. Aliquots, 100 μL each, of the pre-cleared lysate were mixed with 5 μg of antibody protein and incubated overnight at 4°C. At the end of this period, 20 μL of packed Protein G–Sepharose beads were added to each tube and the tubes were incubated for 1 h at 4°C. Following extensive washing with lysis buffer, 50 μL of Laemmli sample buffer (Bio-Rad) was added to each sample and the tubes were heated for 10 min at 95°C. The tubes were centrifuged for 2 min and 25 μL of each sample was loaded onto an SDS-PAGE gel and electrophoresed at 175 volts for 60 min. The electrophoresed samples were electroblotted onto PVDF membranes for Western blot analysis using the murine anti-PSMA antibody 4D8 (5 μg/mL) and developed as described above.

Flow cytometry

LNCaP and PC-3 cells were freshly harvested from tissue culture flasks and a single cell suspension prepared. Approximately 1 million cells were resuspended in PBS containing 0.5% BSA and 50–200 μg/mL of primary antibody and incubated on ice for 30 min. The cells were washed twice with PBS containing 0.1% BSA, 0.01% NaN_3, resuspended in 100 μL of 1:100 diluted FITC-conjugated rabbit-anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), and incubated on ice for an additional 30 min. The cells were again washed twice, resuspended in 0.5 mL of wash buffer, and analyzed for fluorescent staining on a FACSCalibur cytometer (Becton-Dickinson, San Jose, CA) with CellQuest acquisition software.

FITC labeling of monoclonal antibodies

Purified monoclonal antibodies were first extensively dialyzed against 0.3M sodium carbonate buffer, pH 9.5. A stock fluorescein isothiocyanate (FITC) solution was prepared by dissolving 1 mg solid FITC in 1 mL of DMSO. Stock FITC was added dropwise with constant mixing in an amount to provide 50 μg FITC per mg of antibody protein. Once added, the solution was incubated in the dark at room temperature for 1–3. FITC-labeled antibody was isolated by gel filtration on a Sephadex G-10 column equilibrated in PBS.

Antibody inhibition studies

Approximately 1 million LNCaP cells were initially treated with 200 μg/mL purified 1G9, 3C6, 4D4, or irrelevant mouse IgG_1 antibody in PBS for 1 h on ice. After washing, the cells were incubated with 50 μg/mL FITC-conjugated 1G9, 3C6, or 4D4 monoclonal antibodies for 1 h on ice. After washing, the cells were stained with 10 μg/mL propidium iodide and analyzed by flow cytometry on a FACsCalibur with CellQuest software.

RESULTS

Isolation of hybridoma cell clones expressing anti-PSMA antibodies

Spleens from PSMA immunized animals were fused, and subsequent screening identified multiple independent parental wells with PSMA reactive antibody titres. A total of 39 distinct murine hybridomas were obtained after several fusions, and the binding properties of the monoclonal antibodies produced were characterized as previously described.[19] The monoclonal antibodies produced by these hybridomas could be grouped based upon their binding characteristics to PSMA and to PSMA fragments expressed as bacterial fusion proteins. Figure 1 shows a summary of results from mapping the binding of each antibody to regions of the PSMA molecule. These results indicate that a group composed of three antibodies—1G9, 3C6, and 4D4—displayed distinct antigen binding characteristics. A detailed analysis of their properties is described below.

Solid phase ELISA studies

Binding characteristics of anti-PSMA specific antibodies were studied by solid phase ELISA using full-length PSMA and compared to reactivity against bacterially expressed fusion proteins containing portions of the PSMA protein.

Figure 2 shows solid phase binding results for antibodies 1G9, 3C6, 4D4, and 4D8 compared to antibody 7E11.C5 as a positive control. Strong binding to full length PSMA was observed with all antibodies, although this was weaker with 1G9, 3C6, and 4D4 compared to the other antibodies tested. Additional studies indicated significant prep-to-prep variation in binding properties using purified LNCaP cell derived PSMA with these three antibodies (results not shown). Other studies utilizing authentic, purified recombinant PSMA expressed in a

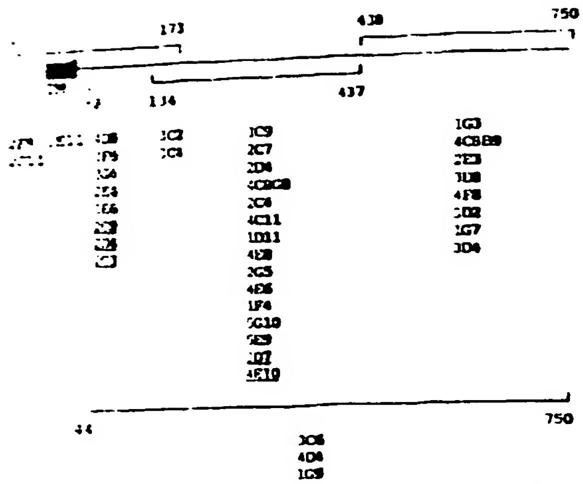

FIG. 1. Diagramatic representation of PSMA and PSMA fragments expressed as bacterial fusion proteins. Full-length PSMA is defined as amino acids 1–750. PSM' is missing the first 57 amino acids, which also contains the transmembrane domain (TM, residues 20–43) of the protein. PSMA fragments utilized are composed of amino acids 1–173, amino acids 134–437, and amino acids 438–750. The antibodies are listed below the region of the protein corresponding to the approximate location of their binding epitope. The lower portion of the figure lists the three antibodies described in this report. Monoclonal antibodies of the IgM isotype are underlined.

mammalian expression system demonstrated strong ELISA reactivity with antibodies 1G9, 3C6, and 4D4 confirming PSMA specificity of these antibodies (results not shown). Strong binding to the fusion protein containing the 1–173 PSMA fragment was observed with 7E11.C5 and 4D8. No binding to any PSMA fragment was observed with any other antibody shown.

Antibody reactivity in a sandwich ELISA for PSMA

A sandwich ELISA utilizing the murine IgM anti-PSMA 4E10-1.14 antibody[19] for capture followed by detection with each IgG is shown in Figure 3. The highest signal was observed with the 7E11.C5 positive control; however, a linear response with the same slope to decreasing antigen concentration was observed for 1G9, 3C6, and 4D4.

Isotype analysis

The isotype of antibodies 1G9, 3C6, and 4D4 was determined to be IgG_1. The isotype of antibody 4D8 was determined to be IgG_{2b}.

Western blot analyses

Western blot analyses were conducted with antibodies 1G9, 3C6, and 4D4 utilizing PSMA from a variety of sources, that is, LNCaP cells, recombinant baculovirus expressed PSMA, and seminal fluid. No reactivity was observed on blots with PSMA from any source using these antibodies (results not shown).

Reactivity with native and denatured PSMA

The inability of this group of antibodies to detect PSMA by Western blot, or to react with PSMA protein fragments by ELISA, suggested protein conformation may be important for antibody binding. This would also be consistent with prep-to-prep variation in binding to purified LNCaP cell PSMA. Experiments were conducted to test the effect of heat denaturation of both purified PSMA and LNCaP cell membranes on antibody binding properties. Figure 4 shows the effect of heat denaturation of isolated PSMA on antibody binding. No effect

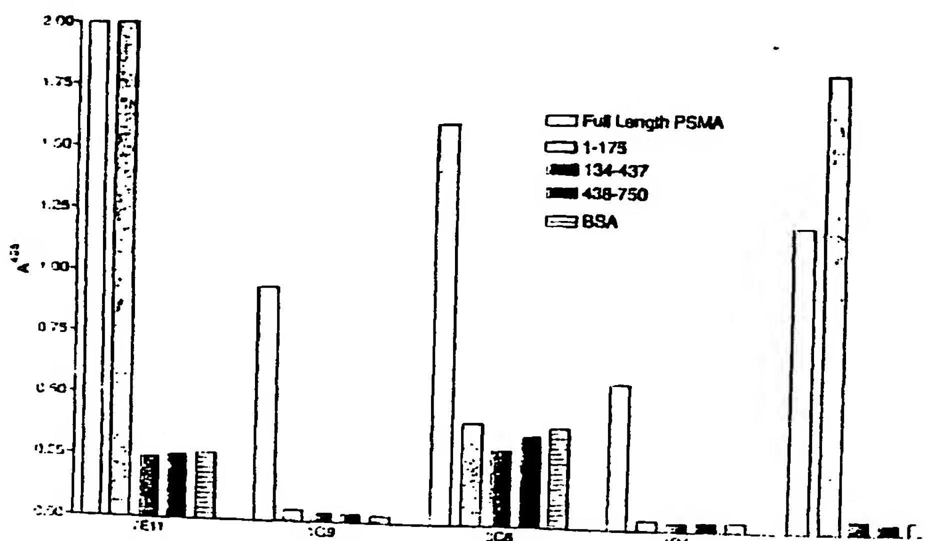

FIG. 2. Solid phase ELISA of full-length PSMA and bacterially expressed fusion proteins containing PSMA fragments. The assays were conducted as described in the text.

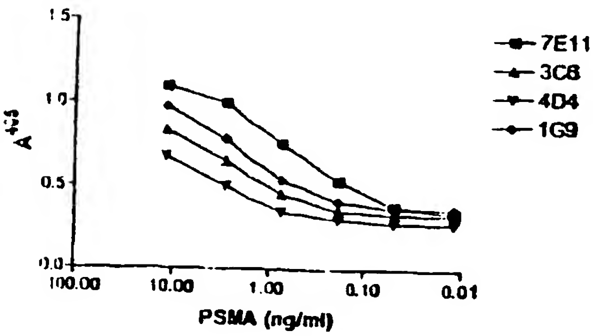

FIG. 3. Sandwich ELISA of immunoaffinity purified PSMA. Wells were coated with 4E10-1.14 anti-PSMA IgM antibody and used to capture purified PSMA present at the indicated concentration. The amount of PSMA bound to the wells was quantitated after incubation of the wells with excess amounts of the indicated IgG_1 antibody.

was observed on binding with antibodies 7E11.C5 or 1G3. Antibody 1G3 has previously been shown to react with a linear epitope in the C-terminal portion of the protein.[19] In contrast, significantly reduced antibody binding was observed with denatured PSMA for antibodies 1G9, 3C6, and, to a lesser extent 4D4.

Results with native and heat denatured LNCaP cell membranes are shown in Figure 5. Significant binding of 1G9, 3C6, and 4D4 to native LNCaP cell membranes was observed. This reactivity was greatly reduced after heat denaturation of the membranes, particularly for antibodies 1G9 and 3C6, wherein detectable binding was only observed at very high antibody concentrations. Once again, 4D4 was somewhat less sensitive than the others to the effect of heat denaturation of the antigen. In contrast, no significant difference was observed with binding of 7E11.C5 to either native or denatured LNCaP cell membranes. Interestingly, although 7E11.C5 consistently showed superior binding to isolated antigen compared to antibodies 1G9, 3C6, and 4D4, the opposite result was obtained when membrane fractions were used (compare Figures 4 and 5). This suggests that the epitope recognized by these antibodies is readily accessible, perhaps distributed on the extracellular portion of the protein.

Immunoprecipitation of native PSMA

Figure 6 shows results of immunoprecipitation of a detergent solubilized lysate from LNCaP cells with antibodies 1G9, 3C6, and 4D4. In this experiment, the lysate was treated with the indicated antibody, the immune complexes were isolated by binding to Protein G-Agarose, and the bound proteins separated by SDS PAGE for Western blots using antibody 4D8. A Western blot of LNCaP cell lysate (lane 1) indicates that 4D8 reacts with both PSMA and PSM' (a 100-kDa soluble variant of PSMA that is missing the first 57 amino acids due to usage of an alternate start site[23]). This indicates that 4D8 recognizes an extracellular epitope within amino acids 58 to 173 of the PSMA protein. Lanes 3–5 show the presence of a strongly stained doublet band corresponding to PSMA and PSM'. No immunoprecipitation of either of these bands was observed with an isotype matched irrelevant mouse antibody (lane 2). Only heavy- and light-chain antibody protein bands bound by the Protein G-Agarose beads were observed. Immunoprecipitation of the band corresponding to PSM' restricts the epitope specificity of each of these antibodies to the extracellular domain of the protein.

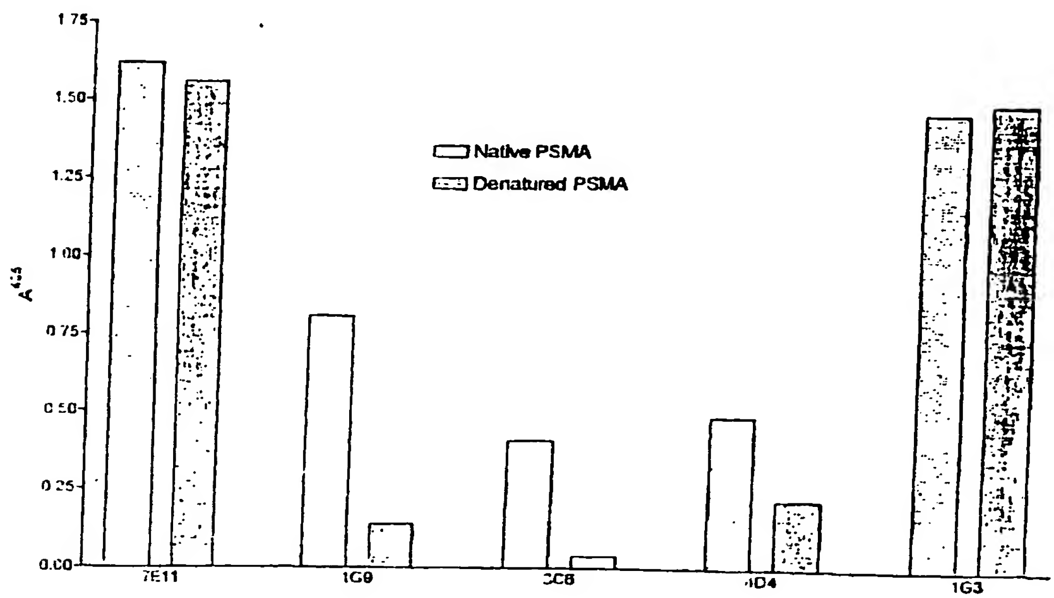

FIG. 4. Effect of heat denaturation of LNCaP cell derived PSMA on antibody binding. The experiments were conducted using linear epitope antibodies 7E11.C5 and 1G3 (specific for an epitope in the C-terminal region of the protein) as controls.

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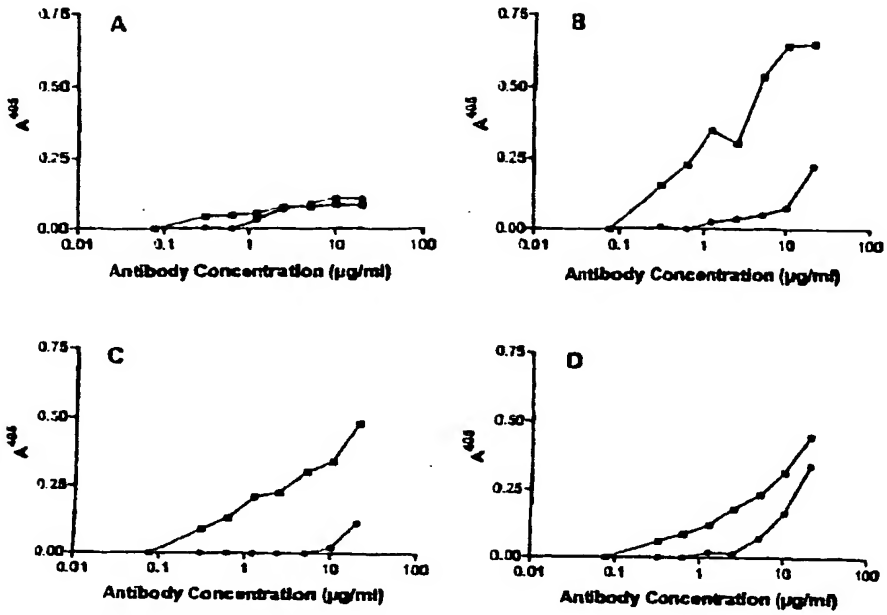


FIG. 5. Effect of heat denaturation of LNCaP cell membranes on antibody binding. The experiments were conducted using the indicated concentrations of antibodies: panel A, 7E11.C5; panel B, 1G9; panel C, 3C6; panel D, 4D4. Results with native membranes are shown by the solid squares; results with heat-denatured membranes are shown by the solid circles.

Flow cytometric analysis of antibody binding to LNCaP and PC-3 cells

Figure 7 demonstrates results from a flow cytometric analysis using 1G9, 3C6, and 4D4 with unfixed LNCaP and PC-3 cells. Strong staining of PSMA expressing LNCaP cells was observed with each antibody (solid histograms). Negative staining was observed with an irrelevant isotype matched murine antibody (open histograms). No staining was observed when PSMA negative PC-3 cells were used with these antibodies (results not shown).

Antibody epitope analysis

Taken together, the results indicate that 1G9, 3C6, and 4D4 are specific for protein conformational epitopes present within the extracellular domain of PSMA. Experiments were conducted to determine if each antibody reacted with the same or different epitopes. Figure 8 shows results of inhibition of antibody binding in a flow cytometric analysis. In these experiments, antibodies 1G9, 3C6, and 4D4 were individually directly FITC-labeled. The effect on staining intensity with these labeled antibodies after prior treatment with unlabeled antibody was determined in a flow cytometric analysis.

Panel A shows results of inhibition of FITC-labeled 1G9. Substantial labeling of LNCaP cells occurred after pretreatment with an isotype matched irrelevant murine antibody (solid histogram) representing maximal antibody binding. Pretreatment with 200 μg/mL of unlabeled 1G9 significantly reduced the extent of FITC-1G9 labeling (brown histogram) representing maximal inhibition in this assay. Pretreatment with 200 μg/mL of 3C6 or 4D4 provided differing results. Essentially no change in FITC-1G9 labeling occurred after 3C6 pretreatment (blue histogram), whereas substantial but incomplete inhibition of FITC-1G9 occurred after pretreatment with 4D4 (green histogram).

Panels B and C show results from similar experiments with FITC-labeled 3C6 and FITC-labeled 4D4. Each antibody was effective in inhibiting itself, representing maximal inhibition. Weak inhibition of FITC-3C6 labeling was observed with 4D4, and 1G9 was found not to be inhibitory to 3C6 (panel B). Antibody 1G9 inhibited FITC-4D4 similarly as did unlabeled 4D4, and 3C6 had no effect of FITC-4D4 labeling of LNCaP cells (panel C). Taken together, these results indicate that 3C6 recognizes a distinct epitope from either 1G9 or 4D4. Antibodies 1G9 and 4D4 most probably react with epitopes in close proximity to each other, perhaps with some overlap.

DISCUSSION

PSMA is a useful marker for prostatic epithelial cells, particularly in cancer. PSMA was initially defined by the monoclonal antibody 7E11.C5.[3] Studies with this antibody have led to the cloning of the gene for the protein[1] and demonstrated high prostate specificity in terms of tissue expression.[24,25] The potential utility of PSMA in clinical applications has led to the production of additional monoclonal antibodies with different binding properties than 7E11.C5.

Previous reports from three different labs described the isolation of murine monoclonal antibodies specific for extracellular portions of the PSMA protein.[11,12,18–20] In general, these antibodies have similar properties in binding to linear amino acid sequence epitopes within the extracellular domain of both

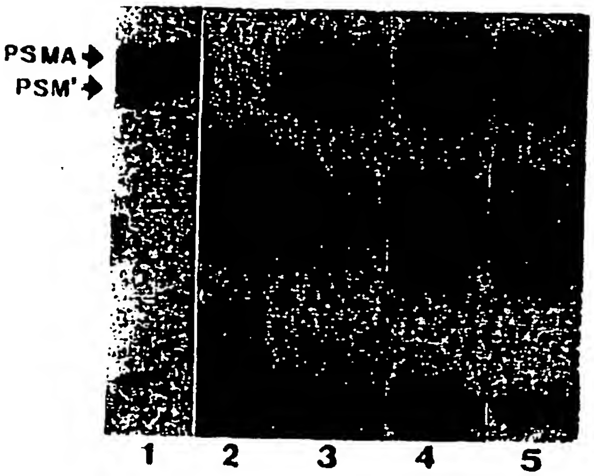


FIG. 6. Immunoprecipitation of PSMA from LNCaP cells. The experiments were conducted using antibody 4D8 to detect immunoprecipitated PSMA in a Western blot. Lane 1, LNCaP cell lysate as Western blot standard; lane 2, immunoprecipitation with irrelevant mouse IgG_1 negative control; lane 3, immunoprecipitation with 1G9; lane 4, immunoprecipitation with 3C6; lane 5, immunoprecipitation with 4D4. The identity of PSMA and PSM' on the blot is indicated.

native and denatured protein. A recent immunohistochemical analysis using a small group of these antibodies has shown an interesting pattern of staining of vascular endothelial cells from a variety of types of malignancies, but not in normal tissue vasculature.[11,12] These results suggest that PSMA-specific antibodies may also have utility in a monoclonal antibody based anti-neovasculature therapy.

The results presented in this paper demonstrate properties of a distinct class of anti-PSMA antibodies, antibodies that do not recognize linear amino acid sequence epitopes but instead bind to protein conformational epitopes. That is, this panel of antibodies appears to recognize native protein epitopes resulting from conformational folding of the PSMA molecule. Presumably, these epitopes arise when amino acids from differing portions of the linear sequence come together in close proximity in three-dimensional space. Data indicates that the epitopes recognized by these antibodies are distributed on the extracellular side of the plasma membrane.

In contrast to monoclonal antibodies specific for linear sequence epitopes present in the PSMA protein,[11,12,18–20] this panel of antibodies does not Western blot PSMA or PSM', nor are they reactive with bacterially expressed fusion proteins containing portions of the PSMA protein. Further, antigen binding properties of these antibodies are greatly diminished by heat denaturation of the antigen. This observation is also in contrast to linear epitope specific antibodies. Studies presented here demonstrate that antibodies 1G9, 3C6, and 4D4 all immunoprecipitate both full length PSMA and PSM', indicating binding specificity within the extracellular domain of the protein.

We have prepared 36 other anti-PSMA monoclonals specific for linear sequence epitopes of the protein and assessed them for their ability to bind to live LNCaP cells by flow cytometry. Although these antibodies efficiently bind PSMA in ELISA or Western blot experiments, they provide a relatively small to moderate shift in fluorescence intensity in a flow cytometric experiment with live LNCaP cells.[19] In each case, 1G9, 3C6, and 4D4 all strongly stain live LNCaP cells suggesting they are particularly well suited to recognizing live prostatic cancer cells *in vivo*.

PSMA has been utilized as a molecular target for prostate cancer detection via *in vivo* scintigraphic imaging using ^{111}In-labeled 7E11.C5 antibody (ProstaScint, Cytogen, Inc., Princeton, NJ).[26,27] This antibody, recognizing an intracellular epitope,[17] is presumed to label apoptotic cells present in larger tumor masses. This limitation gives rise to a rather low sensitivity of the imaging agent *in vivo*. Clearly, a more effective means for localizing prostatic cancer lesions would involve antibodies specific for extracellular epitopes, in particular, antibodies that strongly bind protein epitopes accessible on live prostate cancer cells. It is expected that greater accessibility of an extracellular epitope would improve the efficiency of uptake of labeled diagnostic reagents for imaging metastatic lesions. This would also have application in potential antibody directed

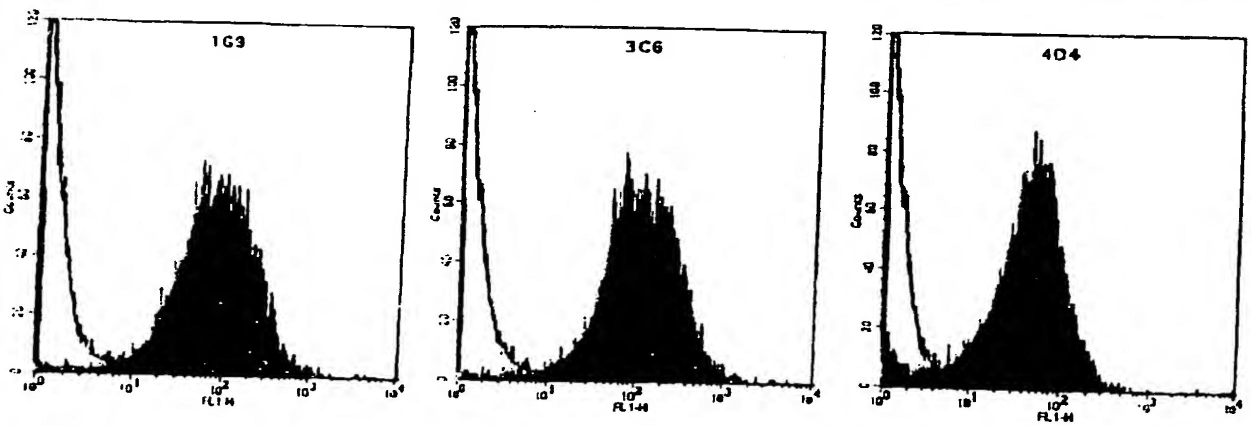
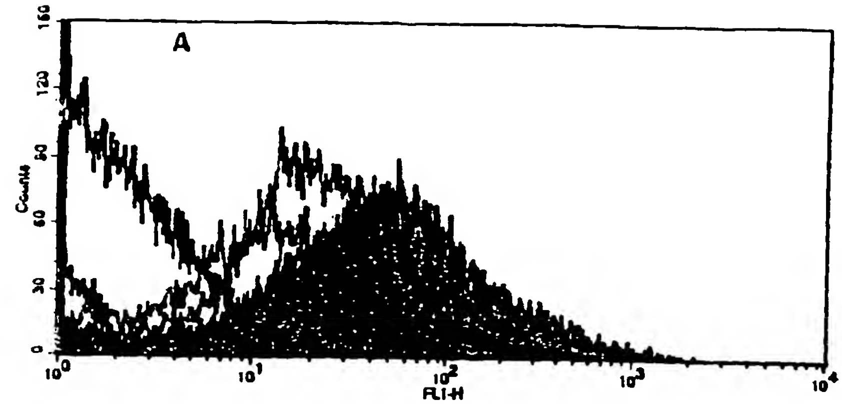
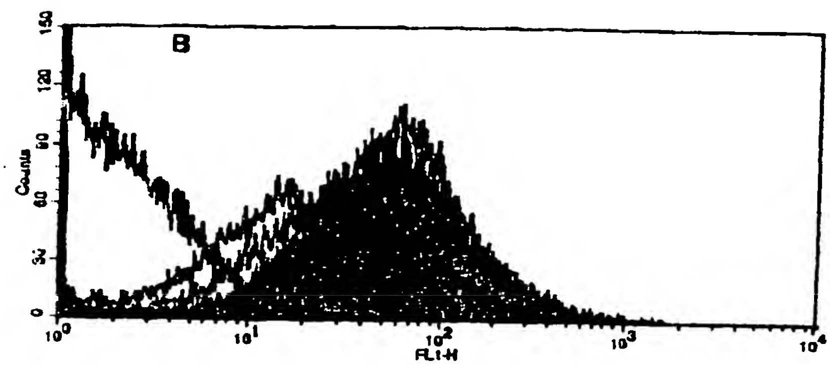


FIG. 7. Flow cytometric analysis of antibody binding to unfixed, live LNCaP (solid histograms) cells. Staining of LNCaP cells with an irrelevant isotype matched antibody is indicated by the open histograms.





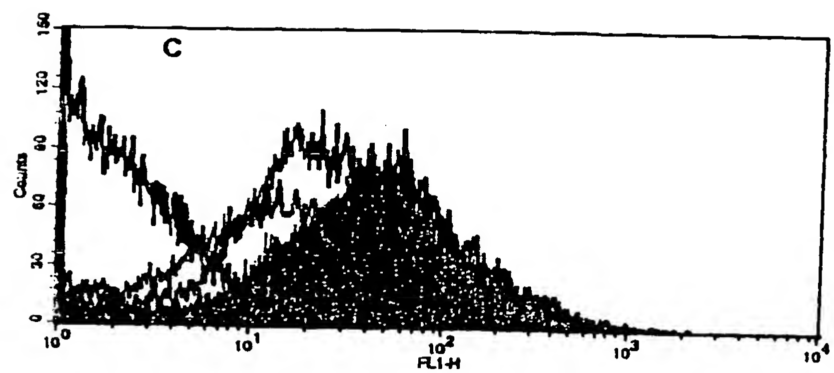


FIG. 8. Inhibition of antibody binding. The effect of pretreatment with either an irrelevant mouse IgG_1 antibody (solid red histograms), 1G9 (brown histograms), 3C6 (blue histograms), or 4D4 (green histograms) on binding of the indicated FITC-conjugated antibody is shown. Panel A, FITC-conjugated 1G9; panel B, FITC-conjugated 3C6; panel C, FITC-conjugated 4D4. Binding of a FITC-labeled, isotype-matched irrelevant mouse antibody to LNCaP cells is shown in the black open histogram. The conditions of the experiment are given in the text.

therapies wherein efficient uptake of antibody by live cells would also be a key element for tumor cell targeting through drug, toxin, radioisotopic immunoconjugates, or bifunctional antibody approaches. Work is underway to define antibody binding characteristics applicable to clinical use.

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Address reprint requests to:
Eric H. Holmes
Northwest Biotherapeutics, Inc.
2203 Airport Way South, Suite 200
Seattle, WA 98134

E-mail: eholmes@nwbio.com

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